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SCREENING METHOD INVOLVING MGDG SYNTHASE

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The present invention relates to a method for screening and for selecting antiparasitic agents (parasites of the apicomplex phylum) and/or herbicides.

Apicomplexes are single-cell parasites responsible for diseases which are among the most serious for the human species: malaria, the primary deadly disease in the world, and toxoplasmosis, one of the two most common opportunist infections in individuals suffering from AIDS. These infectious diseases are spreading, while no treatment at this time makes it possible to eradicate the parasites which cause them: *Plasmodium* which are found in the hepatic cells and red blood cells of individuals suffering from malaria, and *Toxoplasma* which invades, among other things, the brain of individuals suffering from toxoplasmosis.

Specifically, according to the World Health Organization (WHO), malaria affects more than 500 million human beings and causes 2.5 million deaths per year. Malaria kills half the children under the age of 5 in Africa. 40% of the world population live in regions where malaria is present, and these regions spread each year. Pesticide treatments have caused the mosquitoes which are vectors for the parasites (anopheles) to become resistant and the parasite itself (4 species of *Plasmodium*, including *Plasmodium falciparum* for 95% of cases) is becoming increasingly resistant to known treatments (in particular chloroquine derivatives). According to estimates, malaria is the first or second (after diarrhea) deadliest disease in the world. The direct and indirect cost of malaria in Africa has gone from 800 million dollars in 1987 to more than 2 billion dollars in 1998. The resistance to treatments and the spread of the regions where malaria is present make this scourge a major challenge of the 21st century.

According to the National Institute of Health (NIH), toxoplasmosis is the primary brain infection in individuals suffering from AIDS. The parasite (*Toxoplasma gondii*) is common and it may be considered that one person in two has been infected, either by eating incorrectly cooked meat or by coming into contact with domestic cats. Toxoplasmosis is serious only in frail individuals, in particular human fetuses and individuals suffering from AIDS. In the case of AIDS, the patients exhibiting a CD4<sup>+</sup> level < 100/mm<sup>3</sup> develop symptoms of toxoplasmosis, in general by reactivation of a prior infection. The known treatments (sulfadiazine and pyrimethamine the most common, but also clindamycin, azithromycin, clarithromycin, dapsone and atavaquone) must sometimes be prescribed indefinitely since, although they are lethal for the parasite *in vitro*, these substances do not always eliminate the parasite from the body. Since these treatments are sometimes incompatible with tritherapy, prophylaxis is difficult. In the battle which is still to be fought against AIDS, it is therefore fundamental to investigate novel treatments capable of eradicating *Toxoplasma*.

Other apicomplexes, such as those of the *Eimeria* genus, are responsible for coccidiosis in birds and cattle.

Recently, it has become known that these parasites have plant subcellular structures (McFadden et al., *Nature*, 1996, **384**, 482; Köhler et al., *Science*, 1997, **275**, 1485-1489), termed apicoplasts.

These authors have identified, by *in-situ* hybridization, the plast which contains a 35 kb DNA in *Toxoplasma gondii*: it is an organelle limited by 4 membranes, - which is close in evolution to that of green algae. This plast has very rapidly been presented as a weakness of apicomplex parasites (Fichera et Roos,

Nature, 1996, 390, 407-409). These authors have in particular shown that some antibiotics, such as fluoroquinolones and macrolides, inhibit prokaryotic DNA gyrases and block the replication of this 35 kb DNA, which appears to be necessary for the survival of the parasite. More recently, Waller et al. (PNAS, 1998, 95, 12352-12357) have shown that this plast contains a protein known to synthesize fatty acids in plant chloroplasts, acyl carrier protein or ACP. The ACP precursor contains a transit sequence of the chloroplast type, which allows the protein (the mature ACP, or a fluorescent label of the GFP type fused with the transit sequence of the ACP precursor) to be integrated into the parasite plast.

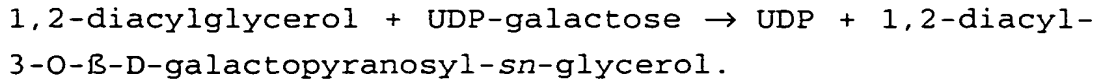
ACP is not, unfortunately, specific for the plant kingdom, and is found in particular in bacteria of the intestinal tract. It does not therefore constitute a specific target for a medicinal product which would affect only the apicomplexes living as parasites in the body.

The aim of the inventors has therefore been to provide a target specific for apicomplex parasites, in order to select novel medicinal products which are effective against said apicomplex parasites.

They have now found that MGDG synthase (an enzyme which is essential for the biogenesis of the plast envelope) may be a target of choice for active principles against *Plasmodium* (malaria), *Toxoplasma* (toxoplasmosis) and *Eimeria* (coccidiosis), and for herbicides.

Specifically, MGDG (monogalactosyldiacylglycerol, figure 1) is known to be in all the plasts analyzed to date: it is the most abundant lipid of plastidial membranes (> 50% of the glycerolipids), is vital to plast biogenesis and cell survival and does not exist in the other membrane systems, in particular in animal

cells (Douce, Science, 1974, 183, 852-853); the biosynthesis thereof is catalyzed in the envelope by a UDP-galactose: 1,2-diacylglycerol 3-β-D-galactosyl-transferase (EC 2.4.1.46), also named MGDG synthase, according to the following reaction:



A subject of the present invention is the use of an MGDG synthase for selecting or screening products which inhibit the activity of MGDG synthase and which can be used as active principles against apicomplex parasites, and in particular those responsible for malaria, for toxoplasmosis and for coccidiosis.

A subject of the present invention is also the use of a plastidial membrane isolated from a plant, for selecting or screening products which inhibit the activity of MGDG synthase and which can be used as active principles against apicomplex parasites, and in particular those responsible for malaria, for toxoplasmosis and for coccidiosis.

A subject of the present invention is also the use of an MGDG synthase for selecting or screening products which inhibit the activity of MGDG synthase and which can be used as herbicides.

A subject of the present invention is also the use of a plastidial membrane isolated from a plant, for selecting or screening products which inhibit the activity of MGDG synthase and which can be used as herbicides.

A subject of the present invention is also a method for screening and for selecting apicomplex antiparasitic agents and/or herbicides, characterized in that it comprises:

*pesticides*

- incubating a substance to be tested with an MGDG synthase and

5 - measuring the specific enzymatic activity, after said incubation.

Inhibition of the enzymatic activity is defined by a decrease in the activity of at least 50%, as a percentage for control activity (activity of the enzyme  
10 treated as the test, but in the absence of inhibitor).

In accordance with the invention, said MGDG synthase preferably has an initial specific activity of between 0.1 and 120  $\mu\text{mol}$  of galactose incorporated/h/mg of  
15 protein; some recombinant MGDG synthases may have a specific activity greater than 120  $\mu\text{mol}$  of galactose incorporated/h/mg of protein.

In addition, in accordance with the invention, the MGDG  
20 synthase is of plant origin (spinach, cucumber or Arabidopsis, in particular) and is selected from the group consisting of the purified or recombinant MGDG synthases A and MGDG synthases B.

25 In accordance with said method, the MGDG synthase/substance to be tested incubation is carried out in an incubation medium containing a buffer adjusted to a pH of between 6 and 9 (MOPS-NaOH, Tris-HCl,  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ , 10 to 250 mM CAPS), in the presence  
30 of detergents (3 to 6 mM CHAPS, or LDAO) of a reducing agent (1-10 mM DTT, or  $\beta$ -mercaptoethanol), of phosphatidylglycerol (0.1-2 mM) and of a salt (KCl or NaCl, 10-300 mM); preferably, said buffer contains 50 mM of MOPS-NaOH, pH 7.8, 4.5 mM of CHAPS, 1.3 mM of  
35 phosphatidylglycerol, 1 mM of DTT, 250 mM of  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  and 250 mM of KCl.

Also in accordance with the invention, the enzymatic activity of the MGDG synthase is measured after

constituting micelles, in accordance with the method described in Maréchal et al. (*J. Biol. Chem.*, 1994, **269**, 5788-5798).

- 5 In accordance with the invention, said apicomplex parasite is selected from the group consisting of *Plasmodium*, *Toxoplasma* and *Eimeria*.

10 A subject of the present invention is also the use of an MGDG synthase inhibitor selected in accordance with the method defined above, for producing a medicinal product against parasites.

15 A subject of the present invention is also the use of an MGDG synthase inhibitor selected in accordance with the method defined above, as a herbicide.

20 Besides the arrangements above, the invention also comprises other arrangements which will emerge from the following description, which refers to examples of implementation of the method which is the subject of the present invention and also to the attached diagrams, in which:

25 - figure 1 represents MGDG (monogalactosyldiacylglycerol);

30 - figure 2 is a comparison of spinach, cucumber and Arabidopsis MGDG synthase; figure 2A corresponds to a comparison of the amino acid sequences deduced from the cDNAs encoding the various MGDG synthases; in this figure, atMGD A and atMGD B correspond to sequences derived from *Arabidopsis thaliana*, csmGD A corresponds to a sequence derived from *Cucumis sativa* and soMGD A  
35 corresponds to a sequence derived from *Spinacia oleracea*. \* and : represent symbols for the identical amino acids and the conserved substitutions, respectively; h1 to h7 correspond to 7 putative

$\alpha$ -helices; figure 2B represents a phylogenic tree of mature MGDG synthases;

- figure 3 corresponds to the identification of the  
5 rMGD A reaction product; figure 3A: separation of the  
polar lipids by two-dimensional thin layer  
chromatography; in this figure, MGDG = monogalactosyl-  
diacylglycerol; DGDG = digalactosyldiacylglycerol;  
TGDG = trigalactosyldiacylglycerol; SL = sulfolipid;  
10 PC = phosphatidylcholine; PG = phosphatidylglycerol;  
figure 3B corresponds to the analysis of the  
galactolipids synthesized *in vitro* by the rMGD A;

- figure 4 illustrates the location of the rMGD A in  
15 *E. coli*;

- figure 5 corresponds to the partial purification of  
the rMGD A; figure 5A: fractionation by hydroxyapatite  
agarose chromatography; figure 5B: SDS-Page analysis of  
20 the fraction eluted from the hydroxyapatite agarose  
column.

It should be clearly understood, however, that these  
examples are given by way of illustration of the  
25 subject of the invention, of which they in no way  
constitute a limitation.

**Example 1: Preparation of an MGDG synthase, from a  
plant**

30

The MGDG synthase is solubilized and purified from the  
envelope of spinach chloroplasts, under the conditions  
set out in Maréchal et al. (C.R. Acad. Sci. Paris,  
1991, **313**, III, 521-528; *J. Biol. Chem.*, 1994, **269**, 8,  
35 5788-5798; *J. Biol. Chem.*, 1995, **270**, 11, 5714-5722).

Specifically:

- the envelope membranes of spinach chloroplasts are purified (see Maréchal et al., *J. Biol. Chem.*, 1995, mentioned above).

The envelope membranes may also be obtained in accordance with the following technique: more precisely, all procedures are carried out at 0°-5°C.

10 The chloroplasts are obtained from 3-4 kg of spinach leaves (*Spinacia oleracea* L.) and purified by isopycnic centrifugation using Percoll gradients. The purified intact chloroplasts are lyzed in a hypotonic medium and the envelope membranes are purified from the lysate by

15 centrifugation in a sucrose gradient.

The envelope membranes obtained are stored under liquid nitrogen, in the medium comprising 50 mM of MOPS-NaOH, pH 7.8 and 1 mM of DTT (dithiothreitol);

- 20
- the MGDG synthase is solubilized and purified from the envelope membranes obtained, as specified above (see Maréchal et al., *J. Biol. Chem.* 1995, mentioned above).

25

The MGDG synthase can also be obtained from cucumber (application JP 10014579 in the name of Kirin Brewery Co. Ltd).

30 In the context of the implementation of the method according to the present invention, it is preferable to use an MGDG synthase having at least a specific activity of 0.1  $\mu$ mol of galactose incorporated/h/mg of protein.



**Example 2: Preparation of a recombinant MGDG synthase and constructs for the overexpression thereof in *E. coli*.**

- 5 1) Cloning and overexpression of a class A MGDG [lacuna] in *E. coli*.

- cloning of the MGDG synthase cDNA:

- 10 A 1647 bp fragment corresponding to the mature protein of the cucumber MGDG [lacuna] cDNA (Shimojima et al., 1997) is used as a probe to screen a  $\lambda$ gt11 library obtained from spinach leaves.
- 15 Before screening, the presence of a homologous mRNA is verified by Northern blot on total RNA from spinach leaves. 320,000 plaques are cultured on *E. coli* Y1090 and transferred onto Hybond-N<sup>+</sup> membranes. The membranes are prehybridized for 2 h at 60°C in a solution
- 20 comprising 2 × SSC, 5 × Denhardt's, 0.5% SDS (w/v) and salmon sperm DNA (0.1 mg/ml<sup>-1</sup>).

- The hybridization is carried out for 16 h at 60°C in the same reagent in the presence of 1 ng of
- 25 [ $\alpha$ -<sup>32</sup>P]dCTP-labeled cucumber DNA. The membranes are washed 3 times for 3 min at room temperature in 2 × SSC, 0.1% SDS (w/v) and twice for 15 minutes at 55°C, and then autoradiographed.

- 30 Two positive clones are then purified by 3 rounds of screening. The phage DNA is extracted and digested with *EcoRI* or *EcoRI* and *BamHI*. The two cDNA inserts are subcloned into the pBlueScript SK<sup>+</sup> plasmid (digested with *EcoRI* or digested with *EcoRI*-*BamHI*), for
- 35 sequencing.

The analysis of the restriction fragment and the sequencing show that the two clones correspond to the same cDNA.

The PCR amplification with primers adjacent to the  $\lambda$ gt11 cloning site reveals inserts of 2.5 and 0.9 kb, respectively. The analysis of the sequence of the inserts obtained by PCR shows that the 0.9 kb insert is identical to the 3' end of the 2.5 kb insert.

Consequently, the longest insert is cleaved with the *Bam*HI/*Eco*RI restriction enzymes, subcloned into the pBlueScript SK+ plasmid (stratagene) and sequenced.

10

The sequence obtained, which comprises 1851 nucleotides, appears to be a chimera. It contains an 807 nucleotide sequence which is highly homologous to the coding end in 3' of the cucumber MGDG synthase cDNA, including the stop codon. This truncated DNA is fused at its 5' end with a partial DNA sequence (1044 nucleotides) homologous to  $\beta$ -endoglucanases. The 5' end of the MGDG synthase cDNA is cloned by rapid amplification of cDNA ends (RACE) using the Marathon amplification kit (Clontech).

20

The spinach leaf cDNA is prepared from polyA+ mRNA and used as a matrix for the PCR amplifications of the 5' end of the MGDG synthase cDNA, in accordance with the manufacturer's instructions. The specificity of the reaction comes from the specific primer CTCATTTGAAGGGCAGTAGCACC (nucleotides 870 to 848) (SEQ ID No. 1) and through "hot start" PCR.

25

This method makes it possible to clone a 1001 bp fragment which is then subcloned into the pBlueScript SK+ plasmid and sequenced on both strands in 3 independent clones, so as to be sure that the Taq polymerase does not introduce any error.

30

The 5' RACE fragment includes an identifiable initiation codon and 131 nucleotides of the 5' untranslated sequence. The clone comprising the complete MGDG synthase cDNA is generated from the

spinach leaf cDNA by PCR, using primers specific for the 3' and 5' ends of the cDNA. The sense primer is as follows: CACACAATATTTCCAATGTATACCCAC (nucleotides -82 to -57) (SEQ ID No. 2).

5

The antisense primer is as follows: GATTATCATTTCCCCTCGCCCTGCC (nucleotides 1672 to 1648) (SEQ ID No. 3).

10 The 1765 bp DNA fragment obtained is subcloned into the pBlueScript SK+ plasmid at the *Sma*I restriction site, and the sequence is verified.

15 The cDNA sequence is shorter than the transcript (2.5 kb) detected by Northern blot analysis, thus indicating that it is not complete.

20 The 2 techniques combined (5'-RACE technique and screening of a spinach cDNA library) make it possible to obtain a 1890 bp sequence which includes a 1569 bp open reading frame encoding a 522 amino acid protein (57.5 kDa) (figure 2) which belongs to the MGDG synthase A family.

25 The analysis of the amino acid sequence shows that this MGDG synthase A contains more nonpolar (56%) than polar (44%) residues, 9 cystein residues and 16 histidine residues which may be involved in the chelation of metals; this protein has a basic isoelectric point (pI = 9.16).

30

## 2) Extraction

35 .extraction of the recombinant MGDG synthase (rMGD A) all the procedures are carried out at 4°C. A pellet of recombinant bacteria (34 mg of protein) expressing the MGDG synthase (7 mg of protein) is resuspended in 50 ml of medium A (6 mM of CHAPS, 50 mM of MOPS-NaOH, pH 7.8, 1 mM of DTT) containing 50 mM of KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> and a

mixture of protease inhibitors (1 mM of PMSF; 1 mM of benzamidine; 0.5 mM of caproic acid). After cell lysis by repeated sonication, the suspension is mixed at 0°C in ice for 30 minutes. The mixture is centrifuged for 15 min at 243 000 g (Beckman L2, SW 40 rotor). The supernatant containing the solubilized proteins (16 mg) is loaded onto a hydroxyapatite ultrogel (IBF-France) column (Pharmacia C10/20, 25 ml of gel), equilibrated with a medium A containing 50 mM of  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ . The proteins are eluted using a gradient of  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (50-275 mM) (in a medium A; flow rate: 30 ml/h; fraction volume: 1.5 ml). The recombinant MGDG synthase is eluted at 275 mM of  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ .

### 3) Overexpression of the spinach MGDG synthase in *E. coli*

#### - Materials and methods

Two mature forms of MGDG synthase are overexpressed in *E. coli*, using the pET-15b plasmid (Novagen) and a plasmid, termed pET-Y 3a, which makes it possible to overcome the problem which comes from the fact that the deduced sequence of the MGDG synthase contains 22 arginine residues, 17 of which are encoded by AGG or AGA, these being codons which are, in fact, used very little in *E. coli*. Specifically, pET-Y 3a was constructed by inserting, into the pET-3a plasmid (Novagen), the arg U (or DNA Y) gene encoding the transfer RNA for arginine, associated with the rare AGA/AGG condons.

These two plasmids are linearized with *Bam*HI and *Nde*I. The PCR-amplified fragments are generated from the complete cDNA clone. The pET-15 plasmid is ligated with a fragment encoding the 417 C-terminal residues of the enzyme, which is amplified by PCR using the following primers:

sense primer: GGAGCATATGGGGGTGAGTGATAATG (SEQ ID No. 4)

and

antisense primer: GTTCTGGATCCTCAAGCAGCACAAAGAGT (SEQ ID No. 5)

and digested with the *Bam*HI and *Nde*I enzymes.

5

Another fragment digested with the *Bam*HI and *Nde*I enzymes, encoding the 424 C-terminal residues of the enzyme, is amplified by PCR using the following primers:

10 sense primer: CTTCACATATGCCTTAATTCCGGGGAGAG (SEQ ID No. 6) and

antisense primer: GTTCTGGATCTCAAGCAGCACCGAGTA (SEQ ID No. 7),

15 and is subcloned into the *Bam*HI-*Nde*I restriction site of the pET-Y3 plasmid.

The first construct allows the expression of a histidine-tagged fusion protein (hMGD A) comprising 437 residues (48.24 kDA). The second construct allows  
20 the expression of a 425 amino acid protein (rMGD A) including an additional initiation methionine corresponding to the ATG codon of the *Bam*HI restriction site. The recombinant proteins are expressed in *E. coli* BI.21(DE3). The bacterial cultures are cultured at  
25 37°C, with vigorous shaking (Certomat, 250 rpm), until an optical density of 0.4 to 0.6 is obtained. Recombinant MGDG synthase expression is induced by adding 0.4 mM of IPTG to the medium and the cultures are incubated for 3 h at 25°C. The bacteria are  
30 pelleted by centrifugation (Eppendorf, 14 000 g, 10 min) and solubilized in a buffer A (50 mM MOPS, pH 7.8, 10 mM DTT, 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF and 0.5 mM caproic acid) in the presence or absence of 0.1% Triton X-100 or in a buffer A with 6M  
35 urea. The soluble and insoluble fractions are separated by centrifugation (Airfuge, 115 000 g, 15 min) and analyzed on SDS-PAGE (12% polyacrylamide gel). The proteins are detected by staining with Coomassie blue.

The hMGD is purified to homogeneity from the bacteria by affinity chromatography based on a metal (NTA, Novagen), followed by desalification through a PD10 column (Pharmacia) equilibrated in a mixture comprising  
5 5 mM imidazole, 0.5 mM NaCl and 20 mM Tris-HCl, pH7.9, in the presence of 6M urea.

The pure recombinant protein (1 mg) is used to obtain a rabbit polyclonal antibody (Eurogentec, Belgium). The  
10 IgG is purified by DEA-trisacryl M (IBF, France) chromatography.

#### - Results

15 In order to minimize the effect of the N-terminal end of the target chloroplast sequence, the spinach cDNA is expressed from the residue leucine 99, which corresponds to the putative cleavage site of the signal peptide of the cucumber MGD A precursor (Shimajima et  
20 al., PNAS, 1997, **94**, 333-337).

Using UDP-[<sup>14</sup>C]gal as substrate, it is possible to measure the MGDG synthase activity in the extracts of *E. coli* expressing the rMGD A, after induction with  
25 IPTG: more than 2 μmol of galactose are incorporated/h/mg of protein. The activity determined in the extracts of *E. coli* containing the histidine-tagged protein hMGD A is of the same order (1.3 μmol of galactose incorporated/h/mg of protein).

30 Only an insignificant fraction of [<sup>14</sup>C]-galactose (less than 0.1 μmol of galactose incorporated/h/mg of protein) is observed in the *E. coli* lipids before induction with IPTG. In addition, no [<sup>14</sup>C]-galactose  
35 incorporation is observed in the control bacteria, which express E37, another inner envelope protein (Teyssier et al., Plant J., 1996, **10**, 903-912). After 3 h of induction with IPTG, an extract of *E. coli* containing the overexpressed rMGD A is incubated in the

presence of UDP-[<sup>14</sup>C]-gal, and the lipids are extracted in order to analyze the reaction products.

5 The lipid extract is analyzed by two-dimensional thin layer chromatography, at the same time as the envelope lipids added to the mixture as a standard (Douce et al., *In Methods in Plant Biochemistry, Lipids, Membranes and Aspects of Photobiology* (Harwood et al. eds, 1990, 4, 71-103, Academic Press, London). Figure 10 3A shows that a single radioactive spot comigrates with the MGDG of origin and is detected by autoradiography. A more extensive characterization of the MGDG was carried out by analyzing the polar groups by two-dimensional paper chromatography; in this case also, a 15 single radioactive spot is detected by autoradiography and comigrates with the glyceryl galactose obtained after deacylation of the envelope MGDG by gentle alkali hydrolysis (figure 3B).

20 These results show that the product formed in *E. coli* is effectively MGDG, which is normally absent in *E. coli* membranes. No other lipid containing galactose is formed, unlike that which is observed after incubating isolated envelope membranes in the presence 25 of UDP-[<sup>14</sup>C]-gal.

MGDG synthase activity is catalyzed by a multigenic family of proteins.

30 The bireactional mechanism of MGDG synthase activity has been studied using very enriched membrane protein fractions, as has its selectivity for various molecular species of 1,2-diacylglycerol (Maréchal et al., *J. Biol. Chem.*, 1994, 269, 5788-5798). Certain structural 35 properties of the catalytic site have been elucidated: the existence of amino acids which are important for the catalysis (Cys, His, Lys) and the association of the enzyme with divalent metals (Maréchal et al., *J. Biol. Chem.*, 1995, 270, 5714-5722). The functional

molecular mass during the inactivation of MGDG synthase has, moreover, been determined by gamma irradiation: the apparent molecular mass of envelope MGDG synthase is  $97 \pm 5$  kDa. Since the mature MGDG synthase polypeptide is close to 45 kDa in size in a denaturing gel, it is probable that, in the envelope, the MGDG synthase is in dimeric form. A functional molecular mass of  $114 \pm 12$  kDa has also been deduced, using the same technique, for purified recombinant MGDG synthase.

5

10 A. This result suggests that MGDG synthases are probably homodimers.

**Example 3: Measurement of the enzymatic activity, using micelles**

15 The activity of the MGDG synthase is measured on various types of sample, depending on the model chosen: plastidial membrane, membrane fractions of *E. coli* overexpressing a recombinant MGDG synthase (rMGD A,

20 0.7  $\mu$ g protein/assay, enzyme extracted beforehand from a plant (see Example 1)).

**. preparation of micelles**

25 1.3 mM of phosphatidylglycerol (PG) and 160  $\mu$ M of diacylglycerol (DAG) are dissolved in chloroform. After evaporating the solvent under argon, 200  $\mu$ l of incubation medium containing 50 mM of MOPS-NaOH, pH 7.8, 4.5 mM OF CHAPS, 1 mM of DTT, 250 mM of

30  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  and 250 mM of KCl are added and the medium is mixed vigorously so as to resuspend the lipids. 100  $\mu$ l of fractions containing the MGDG synthase, in the incubation medium, are introduced then the medium is again mixed vigorously and then maintained at 20°C

35 for 1 h.

This procedure makes it possible to obtain micelles, in accordance with Maréchal et al., 1994, mentioned above.



.enzymatic reaction

The reaction in the incubation mixture is then initiated by adding 1 mM of UDP-[<sup>14</sup>C]gal (37 Bq/μmol). After 10 min to 1 h, the reaction is stopped by adding  
5 a chloroform/methanol mixture (1:2, v/v), the lipids are extracted in accordance with the method of Bligh et al (Can. J. Biochem. Physiol, 1959, 37, 911-917) and the radioactivity of the labeled galactolipids is determined by liquid scintillation counting as  
10 described in Covès et al. (FEBS lett., 1986, 208, 401-406). The activity is expressed in μmol of galactose incorporated/h/mg of protein.

A high specific activity, i.e. up to 115-120 μmol of  
15 galactose incorporated/h/mg of protein, and even more, can be obtained in a sample rich in MGDG synthase.

3) Specific activity of the overexpressed soluble  
rMGD A

20 When the expression of the spinach MGDG synthase in *E. coli* is analyzed, it is observed that most of the protein (rMGD A) is insoluble and that detergents (6 mM of CHAPS or 1% of Triton X-100) only partially  
25 solubilize the protein (figure 4). On the other hand, almost all the overexpressed protein is solubilized by urea, indicating that most of the MGDG synthase is present in inclusion bodies (figure 4). In this fraction, the activity of the MGDG synthase is very low  
30 (0.03 μmol of galactose incorporated/h/mg of protein). In fact, the hydroxyapatite chromatography analysis of the *E. coli* fractions solubilized by CHAPS shows that only a small fraction (approximately 0.1%) of the recombinant protein synthesized by the bacterium is  
35 active. The experimental conditions used are the same as those used for the envelope MGDG synthase (see above).

In figure 4, the rMGD A expression is induced with 0.4 mM of IPTG as specified in Materials and methods above.

5 Most (50 to 80%) of the activity loaded at the top of the column is found in a narrow peak which is eluted with 275 mM phosphate (figure 5A). In this peak, the specific activity of the MGDG synthase is very high: 115  $\mu$ mol of galactose incorporated/h/mg of protein.

10

The analysis of the polypeptides present in the various fractions shows that a 45 kDa polypeptide corresponding to the rMGD A is present in the active fractions, but also in the dead volume, in which most of the protein is present in an inactive form (figure 5B). This shows that only a fraction (1%) of the protein solubilized by CHAPS is effectively active.

20 In this figure 5B, 20  $\mu$ l of fraction eluted from the hydroxyapatite column are analyzed by SDS-PAGE (12% polyacrylamide gel); the proteins are detected by staining with Coomassie blue; Lo: sample loaded at the top of the column; 15, 30, etc.: fractions eluted from a column; MW: molecular weight marker (Biorad); the rMGD A is indicated with an arrow and the active rMGD A is found only in fraction 67 to 71.

30 **Example 4: Comparison of the biochemical properties of the overexpressed MGD A, with the chloroplast envelope MGDG synthase**

35 The analysis of the activity of the MGDG synthase partially purified from spinach leaf chloroplasts (Maréchal et al., J. Biol. Chem., 1995, **270**, 5714-5722) demonstrated that DTT can protect the activity of the enzyme against oxidation and that N-ethylmaleimide (NEM) and orthophenanthroline are powerful inhibitors of the enzyme..

The overexpressed spinach MGDG synthase has the same properties.

5 The rMGD A, purified by hydroxyapatite chromatography, is very active in the presence of DTT. If the DTT is removed by chromatography on a Biogel P6-DG column, the MGDG synthase loses 85% of its activity, whereas the addition of DTT maintains its activity.

10 The fractions of partially purified rMGD A are desalified by chromatography on a Biogel P6-DG (Bio-Rad) column (Pharmacia, C10/40 column, 30 ml of gel) equilibrated in DTT. Aliquots (200  $\mu$ l) of the fractions are incubated for 40 minutes at 25°C with gentle  
15 stirring, in the presence or absence of DTT. The galactosylation activity is then measured as specified above (see Example 3).

The results obtained are summarized in Table I below:

20

Table I

Fraction purified on hydroxyapatite	Activity (%)
Not desalified	100
Desalified	15
Desalified + 1 mM DTT	75
Desalified + 10 mM DTT	65

25 The activity is expressed as a percentage of the control (not desalified) activity.

Table II shows that the rMGD A is very sensitive to NEM and that protection of the activity is obtained by preincubation in the presence of DAG and/or of PG.

Table II

Pre-incubation (30 min)	Incubation +/- 150 $\mu$ M NEM (10 min)	Incubation +/- 10 mM DTT (10 min)	Enzymatic reaction	Activity (%)
DTT	-	-	+PG+DAG+UDP-gal	100
-	-	+	+PG+DAG+UDP-gal	101
-	-	-	+PG+DAG+UDP-gal	37
-	+	+	+PG+DAG+UDP-gal	35
DTT	+	-	+PG+DAG+UDP-gal	108
UDP-gal	+	+	+PG+DAG	32
PG	+	+	+DAG+UDP-gal	56
PG+DAG	+	+	+UDP-gal	60

In order to obtain the results summarized in Table II, the rMGD A is desalified by chromatography on a Biogel P6-DG (Bio-Rad) column (Pharmacia, C10/40, column, 30 ml of gel) equilibrated in DTT. Aliquots (200  $\mu$ l) of the fractions are incubated for 30 minutes at 25°C with gentle stirring, followed by a 10 min incubation in the presence or absence of 150  $\mu$ M NEM and then a 10 min incubation in the presence or absence of DTT. The galactosilylation activity is then measured as specified above (see Example 3).

The activity is expressed as a percentage of the control activity, i.e. after incubation for 50 min in the presence of 10 mM DTT.

It is also observed that the overexpressed rMGD is inhibited by the hydrophobic chelating agent orthophenanthroline, as shown in Table III below:

Table III

Conditions	Other additions	Activity (%)
Initial activity (time 0)	-	100
Without orthophenanthroline	-	82
	PG	87
	PG + DAG	78
	UDP-gal	72
With orthophenanthroline	-	43
	PG	27
	PG + DAG	92
	UDP-gal	17

The inactivation of the rMGD A by orthophenanthroline is blocked by DAG, but is not affected by UDP-gal.

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As emerges from the above, the invention is in no way limited to its methods of implementation, preparation and application which have just been described more explicitly; on the contrary, it encompasses all the variants thereof which may occur to a person skilled in the art, without departing from the context or from the scope of the present invention.

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